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Production and characterization of a transgenic bread wheat line over-expressing a low-molecular-weight glutenin subunit gene

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Abstract

The end-use properties, and thus the value, of wheat flours are determined to a large extent by the proteins that make up the polymeric network called gluten. Low molecular weight glutenin subunits (LMW-GS) are important components of gluten structure. Their relative amounts and/or the presence of specific components can influence dough visco-elasticity, a property that is correlated with the end-use properties of wheat flour. For these reasons, manipulation of gluten dough strength and elasticity is important. We are pursuing this goal by transforming the bread wheat cultivar Bobwhite with a LMW-GS gene driven by its own promoter. Particle bombardment of immature embryos produced several transgenic lines, one of which over-expressed the LMW-GS transgene. Southern blots confirmed that the transgene was integrated into the wheat genome, although segregation analyses showed that its expression was sometimes poorly transmitted to progeny. We have determined that the transgene-encoded LMW-GS accumulates to very high levels in seeds of this line, and that it is incorporated into the glutenin polymer, nearly doubling its overall amount. However, SDS sedimentation test values were lower from the transgenic material compared to a non transgenic flour. These results suggest that the widely accepted correlation between the amount of the glutenin polymers and flour technological properties might not be valid, depending on the components of the polymer.

Abbreviations: ACN – acetonitrile; HMW-GS – high molecular weight glutenin subunits; LMW-GS – low molecular weight glutenin subunits; RP-HPLC – reversed phase high performance liquid chromatography; SE-HPLC – size exclusion high performance liquid chromatography; TFA – trifluoroacetic acid

Introduction

Wheat flour technological properties are dependent on the type and amounts of storage proteins, made up mainly of gliadins and glutenins. The former are monomeric polypeptides, whereas the latter have a polymeric structure whose individual components, the glutenin subunits, are stabilized by intra- and inter-molecular disulfide bonds (Shewry and Tatham 1997). Glutenin subunits are classically divided into low- (LMW-GS) and high-molecular-weight (HMW-GS) subunits, with LMW-GS more abundant than HMW-GS, being present in about a 6:1 molar ratio (Kasarda 1989). Glutenin polymer size has been found to be directly correlated with flour rheological properties (Dachkevitch and Autran 1989; Gupta et al.

1995), but the structure of the glutenin polymer is poorly understood, due to its large size, exceeding 1,000,000 daltons (Wrigley 1996). HMW-GS of both the *x*- and *y*-types interact with one another (Tao et al. 1992) and with LMW-GS (Keck et al. 1995) to form the glutenin polymers. On the basis of their functional role, glutenin subunits can be considered 'chain branchers' when three or more cysteine residues are available to form intermolecular disulfide bonds, 'chain extenders' when there are two cysteine residues available and 'chain terminators' when the presence of a single free cysteine residue would halt the growing glutenin polymer. HMW-GS are likely to behave as chain extenders and/or branchers, according to their type (Kasarda 1999); typical LMW-GS (LMW-m and LMW-s types, Lew et al. 1992; Masci et al. 1998) should be chain extenders (Shewry and Tatham 1997; Masci et al. 1998; Kasarda 1999), whereas gliadins that have acquired or lost a cysteine residue by mutation (LMW-GS type C and D) might behave as chain terminators (Masci et al. 1993; Masci et al. 2002). Particular subunits, such as HMW-GS 1D*x*5, are likely chain branchers because they show an additional cysteine residue with respect to the other *x* subunits (Kasarda 1999). Glutenin subunits such as HMW-GS 1D*x*5 have the ability to form larger glutenin polymers, with positive consequences on gluten visco-elastic properties (Gupta and MacRitchie 1994). However, when bread wheat was transformed with 1D*x*5 subunit, over-expression of the transgene had a negative effect on gluten properties. In fact, these doughs were over-strong, likely due to excessive chain branching caused by the presence of a large amount of the HMW-GS 1D*x*5 (Rooke et al. 1999; Popineau et al. 2001). An improvement on gluten properties was instead obtained when transformation of bread wheat was performed with 1A*x*1 subunit (Popineau et al. 2001), which is likely to be a chain extender.

Allelic variation of HMW-GS is the major determinant of visco-elastic characteristics of bread wheat, but LMW-GS have also been shown to influence such properties (Gupta et al. 1989; Payne 1987; Payne et al. 1987). Typical LMW-GS are encoded by multigenic families located on the short arm of chromosomes group 1 (*Glu-A3, Glu-B3* and *Glu-D3* loci in bread wheat) and include more than thirty components (Lew et al. 1992). Because LMW-GS are the most common polypetides in the glutenin polymer and their relative amount and/or allelic forms can influence the visco-elasticity of gluten dough, we are investigating the possibility of manipulating gluten strength and elasticity by altering the relative ratio between glutenin subunits and gliadins, by increasing the LMW-GS fraction. Here we present the results of analyses of a line that was derived from the bread wheat cultivar Bobwhite by transformation with a LMW-GS gene driven by its own promoter and that over-expressed the transgene. The rationale of this experiment was that over-expression of a LMW-GS might allow formation of lower molecular weight polymers compared to those resulting from overexpression of HMW-GS even if cross-linking properties were not altered. In fact, while both LMW-GS and HMW-GS can be chain extenders when two of their cysteine residues are not involved in intra-molecular disulfide bonds, the former are smaller, and thus may alter glutenin polymer size less dramatically than HMW-GS (He et al. 1999; Popineau et al. 2001; Vasil et al. 2001).

Materials and Methods

Materials

Immature embryos of the bread wheat cultivar Bobwhite were used for transformation experiments. UBI:BAR (Cornejo et al. 1993) and pLMWF23A plasmid DNA clones, the former conferring resistance to the herbicide BASTA, and the latter containing a LMW-GS gene coded at the *Glu-D3* locus isolated from the bread wheat cultivar Cheyenne (Cassidy et al. 1998), were used for wheat transformation. The pLMWF23A clone contains, in the *Eco*RI site of pBluescript (Stratagene, La Jolla, CA), the coding region of a LMW-GS gene flanked by about 1200 bp and 1600 bp of the 5' and 3' flanking regions, respectively (Cassidy et al. 1998). The coding region, including the signal peptide, is 921 bp and the molecular weight of the deduced protein is 32,842.

Plant transformation

UBI:BAR and pLMWF23A plasmid DNAs (12.5 μg each) were co-transformed into wheat using microprojectile bombardment as described by Blechl and Anderson (1996). Transformation experiments were performed on 1250 immature embryos. Transformants were identified by their resistance to bialaphos in tissue culture.

SDS-PAGE of progeny and determination of the presence of the transgenic polypeptide in the polymeric fraction

Half seeds (10-45 from each of the eleven lines obtained) with embryos removed, were crushed and extracted with SDS-PAGE sample buffer (Tris-HCl 0.07M. pH 6.8, 2% SDS, 10% glycerol, 1.5M dimethylformamide, 1% DTT, 0.02% pyronine-Y as a tracking dye). Aliquots (3 μl) of each extract were loaded on an SDS-PAGE (T = 12, C = 1.28 in the main gel and T = 3.75, C = 2.67 in the stacking gel). For each gel the endosperm proteins extracted from cultivars Bobwhite and Cheyenne were run as reference genotypes. Electrophoresis was performed in a Hoefer™ SE600 apparatus (Hoefer, South San Francisco, CA) at 30 mA per gel, with cooling, and the run was stopped one hour after the tracking dye was off the gel. Staining was performed according to Neuhoff et al. (1988).

Seeds over-expressing the LMW-GS transgene were further analyzed to determine if the transgenic product was incorporated into the polymeric fraction. Half seeds from two samples (T_2 generation) were crushed, and the monomeric and oligomeric fractions were extracted twice with 500 μl of 50% propan-1-ol (soluble fraction). Aliquots corresponding to the soluble fraction were analysed by SDS-PAGE as described above, under reducing and non-reducing conditions (by removing DTT from the SDS sample buffer). The residue remaining after removal of the soluble fraction (insoluble fraction) was extracted with SDS-PAGE sample buffer and aliquots loaded on the same SDS-PAGE used for the soluble samples.

Quantification of the transgene product

Over-expression of the transgene was quantified by densitometric analysis of the SDS-PAGE pattern of total endosperm proteins and was performed on five seeds of cultivar Bobwhite (wild type) and five T_3 seeds, by loading 3 μl of each sample extracted as described above. Densitometric analysis was performed using the program Kodak Digital Science 1D (Kodak).

Quantification was also performed by Reverse Phase High Performance Liquid Chromatography (RP-HPLC) on the residue fraction. Transgenic and wild type seeds were crushed, and the soluble fraction extracted with 50% propanol as described above. The residue was extracted in a 1:10 ratio (mg μl^{-1} with respect to initial weight) with a buffer containing 50% propanol, 50 mM Tris pH 8.8, 1% DTT, 4M urea for 1 hour at room temperature. After centrifugation, the supernatant was combined with an equal volume of 50% acetonitrile (ACN) containing 0.1% trifluoroacetic acid (TFA), filtered through a 0.22 μm filter and injected (50 μl) into an RP-HPLC system (System Gold Beckman apparatus, composed of the solvent delivery module 126 and UV detector module 166) equipped with a Vidaq C_8 column (4.6 mm × 25 cm) and a pre-column cartridge (RP-300, 30 × 4,6 mm, Aquapore, Brownlee). The solvents used were (A) 0.1% TFA and (B) ACN containing 0.1% TFA, and separation was performed with a linear gradient of 30-45% B in 70 min, at a flow rate of 1 ml min^{-1}. Detection was by UV absorbance at 210 nm and the column was equilibrated at 50 °C. Peaks were collected, dried down, and analysed by SDS-PAGE as described above. Over-expression of the transgene was quantified by comparing the area of the peak corresponding to the transgenic LMW with a peak of equivalent retention time for cv. Bobwhite (wild type).

Determination of the polymerization behaviour of the transgenic polypeptide

Two-dimensional analysis (unreduced vs. reduced) of the soluble fraction

This analysis was performed in order to verify the presence of the transgenic protein in the oligomeric fraction. Seeds of cv. Bobwhite (wild type) and of the transgenic line were crushed and the soluble fraction extracted as described above. Aliquots of 100 μg were dried down and re-dissolved in 10 μl of non-reducing SDS-PAGE sample buffer (as described above, with DTT omitted). The first dimension of electrophoresis was performed in a Mini-Gel Apparatus (Bio-Rad). The SDS-PAGE gel (as described above) was 0.75 mm thick. Electrophoresis was performed at 200 V until the dye reached the bottom of the gel. The first dimension gel was stained according to Neuhoff et al. (1988). The lanes corresponding to wild type and the transgenic line were cut out, equilibrated for 20 min at room temperature in equilibration buffer (32.5 mM Tris, pH 6.8, 2% SDS, 50% glycerol, 1% DTT, pyronine-Y) and layered on the top of the second dimension gel that differed in thickness (1 mm) from the first dimension gel. Electrophoresis conditions and staining were as described above.

Partial reduction of the polymeric fraction

Partial reduction was performed on the residue fraction of the transgenic material (Werner et al. 1992), in order to study the pattern of polymerization of the transgenic polypeptide. Residue was obtained by removing soluble polypeptides with 50% propanol as described above. Partial reduction was performed on the same residue, by sequential extraction (10 μl mg^{-1}) with SDS-PAGE buffer containing DTT 0.05 mM, 0.5 mM, 1 mM, 5 mM. Extractions were performed for 30 min at 65 °C. Five μl were loaded on a mini-gel apparatus (Bio-Rad) (T = 11 and C = 2.67 in the main gel; stacking gel as described above; 0.75 mm tickness) and electrophoresis performed at 200 V until the dye reached the bottom of the gel. Staining was performed according to Neuhoff et al. (1988). Lanes corresponding to the four partially reduced samples were cut out of the gel, equilibrated and run on a second dimension gel, as described in the previous section.

Endoproteinase Lys-C digestion of the polymeric fraction

When endoproteinase Lys-C is used on intact glutenin polymers, only HMW-GS are digested, because lysine residues present in LMW-GS are not accessible (Tao et al. 1992). This allows solubilization of lower size glutenin polymers that can be analyzed in more detail. Residues were obtained as described above starting from 60 mg of transgenic flour. Lys-C (Boeheringer Mannheim, Germany) digestion was performed as reported in Tao et al. (1992), with the exception that 10 mM iodoacetamide was added to digestion buffer in order to avoid possible disulfide exchanges during stirring. Digested material was precipitated with cold acetone for 1 h at 4 °C. The pellet was collected by centrifugation, dried and resuspended in 80 μl of SDS-PAGE sample buffer without reducing agent. Twenty μl were loaded on a Mini-Gel Apparatus (Bio-Rad) (T = 11, C = 2.67 in the main gel; stacking gel as described) for the first dimension electrophoresis, and run under the same conditions as described. The lanes containing digested polymers were equilibrated and ran on a second dimension gel as earlier described.

Size Exclusion High Performance Liquid Chromatography (SE-HPLC)

In order to evaluate the effect of the presence of the transgenic product on the amount of glutenin polymers, 10 half-seeds of the T_4 generation that did not show expression of the transgene (non-expressors), that had the same electrophoretic pattern as the wild type (grown under the same conditions as the transgenic plants over-expressing the LMW-GS), and 10 half seeds belonging to the same generation, but over-expressing the transgenic LMW-GS (expressors), were picked randomly. Each set of seeds was crushed to flour and mixed, in order to avoid possible differences in protein content among different seeds.

The propanol soluble fractions, obtained as described above, were analyzed by mixing 20 μl with 200 μl of elution buffer (50 mM Tris-HCl, pH 6.8, 2% SDS, 4M Urea) and injecting the entire sample onto a BioSep-SEC-S 4000 (Phenomenex, Torrance, CA), 7.8 mm × 30 cm column equilibrated at 40 °C and equipped with an on-line pre-column cartridge (Security Guard, Phenomenex). Separation was performed in 40 min at 0.7 ml min^{-1} with a Beckman HPLC system (Pump 110, Detector 167) and detection was at 280 nm.

The insoluble fraction was extracted from the dry residue with the elution buffer (added in proportion 1 μg:20 μl, dry weight) for 1 h at room temperature by gentle agitation. After two centrifugations at 14,000 x g, 20 μl were injected and SE-HPLC was performed as for the soluble fraction.

In both cases (soluble and insoluble fractions), three replicates were performed. Peak areas were calculated after subtracting a horizontal baseline corresponding to the elution buffer baseline.

Protein content and SDS sedimentation test

Seeds corresponding to T_3 and T_4 generations, that showed either the same electrophoretic pattern as ('non-expressors' (see Figure 1, lane 1) or those with the highest over-expression of the LMW-GS transgene ('expressors', see Figure 1, lanes 2-6), were crushed, mixed in 1:2 ratio (T_3:T_4) and 1 g was used to determine protein content by the Kjeldahl method (N × 5.7). The same samples (1 g) were submitted to SDS sedimentation test according to Dick and Quick (1983). Three replicates were performed. The same procedure was applied to T_4 seeds that did not show expression of the transgene, but had identical electrophoretic pattern as non-expressors (see previous section). Both expressors and non-expressors were grown under the same conditions.

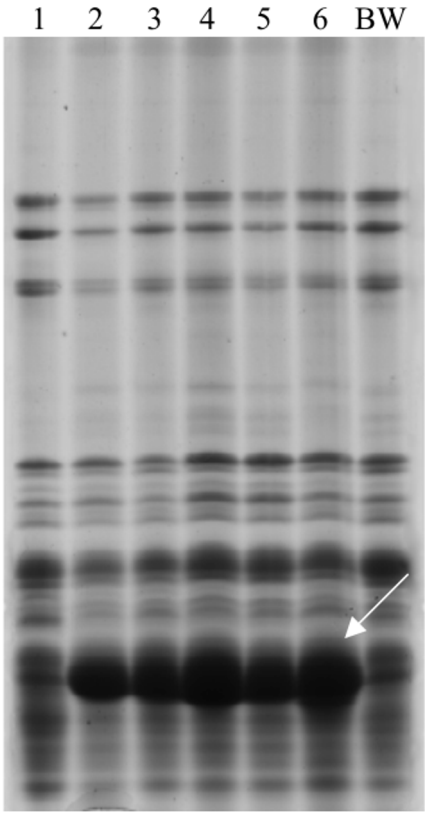


Figure 1. SDS-PAGE of protein extracts from different T_1 seeds (1-6) belonging to the line showing over-expression of the transgenic LMW-GS. A seed protein extract from untransformed cv. Bobwhite (BW) is included for comparison. The arrow shows the position of the transgenic LMW-GS.

Southern Blotting

Genomic DNA was extracted according to D'Ovidio et al. (1992) and 5-15 μg were digested with *Eco*RI, in order to release the entire LMW-GS fragment contained in the pLMWF23A clone used in the transformation experiments. Fractionation was performed on a 1.2% agarose gel and the DNA transferred to a nylon membrane (Roche) following standard procedures. Southern blot analyses were initially performed with both pLMWF23A and pLMW21M18 clones, the latter corresponding to a LMW-GS gene isolated from the durum wheat genotype Line 21 (D'Ovidio et al. 1999). These clones share an almost perfect sequence identity, with the pLMW21M18 having a shorter repetitive region. Both probes gave the same hybridization pattern, but, since the pLMW21M18 produced hybridizing bands with lower background, it was chosen for subsequent hybridization experiments.

The probe pLMW21M18 (50-100 ng), was labelled with digoxigenin by PCR for 30 amplification cycles in a 50 μl total volume using 0.5 μl of Taq DNA polymerase (Amersham), 1X reaction buffer, 50 μM of each dATP, dCTP and dGTP, 40 μM dTTP, 10 μM Dig-11-UTP(Digoxigenin-11-uridine-5'-triphosphate) and 60 ng each of specific primers amplifying the entire coding region. The denaturing step was at 94 °C for 1 min, the annealing step was at 60 °C for 1 min and the extension step was at 72 °C for 1 min. Prehybridization and hybridization reactions were carried out at 65 °C in a solution containing 5X SSC (0.75M NaCl, 0.075M sodium citrate pH 7), 0.02% (w v^{-1}) SDS, 0.1% (w v^{-1}) N-lauroylsarcosine and 1% (w v^{-1}) blocking reagent (Boehringer). Filters were prehybridized for 3 hours and then hybridized for 6 hours at 65°C with digoxigenin-labelled clones. After hybridization, filters were washed twice at 65 °C in 2X SSC plus 0.1% SDS for 15 min each. Immunological detection was performed using the chemoluminescent system CSPD Ready-To-Use (Roche), according to the manufacturer's instructions.

Results

Genetic transformation and over-expression of the transgenic LMW-GS

Eleven independent lines were selected based on their ability to grow on culture media containing 3 mg l^{-1} of bialaphos. Because it was not possible to verify the presence of the transgene by PCR, due to its high sequence similarity with endogenous LMW-GS, all the selected lines were checked for the expression of the transgenic LMW-GS by SDS-PAGE analysis of the proteins extracted from endosperm tissue of T_1 seeds. Of the eleven selected plants, only one showed detectable over-expression of the transgenic LMW-GS encoded by pLMWF23A (Figure 1). Expression levels varied among the T_1 seeds from this plant, from zero (Lane 1) to very high (Lane 6). The variability in expression is expected since the transgene is segregating in the T_1 generation and individual endosperms could have 0, 1, 2 or 3 copies.

T_1 seeds that showed over-expression were grown for three further generations after selfing. Table 1 reports the number of seeds analyzed in each generation along with the number and percentage of transgenic seeds. In each generation, about 1/3 of the

Table 1. Proportion of transgenic seeds, as evaluated by accumulation of the transgene-encoded protein band in SDS-PAGE

Generation	Total seeds analyzed	No. Transgenic seeds	% transgenic seeds
T_2	30	8	27
T_3	195	77	39
T_4	275	73	26
Total	500	158	32

progeny inherited transgenes with detectable expression. This is significantly lower than the 3/4 expected.

The presence of the transgene in the wheat plants over-expressing the LMW-GS was further analyzed by Southern Blotting. Hybridization of *Eco*RI digested genomic DNA to the pLMWF23A-derived probe detected the expected 3.7 kbp fragment derived from the plasmid used in the transformation experiment. This hybridizing signal is far more intense than the others in the profile and it is absent or at least very weak in the recipient cultivar Bobwhite (Figure 2A). The bands shared by the transgenic and wild type Bobwhite are likely to be derived from cross-reacting of the probe with native LMW-GS genes. Bands unique to the transgenic DNA are indicative of transgene copies that have been rearranged. Comparison between digested and undigested genomic DNA from the transgenic wheat line showed that the transgene was integrated into high molecular weight genomic DNA (Figure 2B).

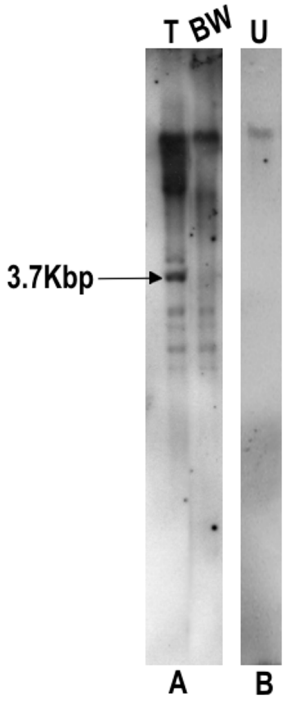


Figure 2. Southern blot analysis of genomic DNA extracted from wild type (BW) and a transgenic sample (T), hybridized with a digoxigenin-labelled LMW-GS sequence. (A) *Eco*RI digested samples; (B) undigested genomic DNA from the transgenic sample. The strong hybridization signal of 3.7 kbp corresponding to the expected fragment contained in the pLMWF23A clone used for transformation is present only in the digested DNA from the transgenic plant.

Quantification of over-expression in endosperm and in residue protein

SDS-PAGE patterns of total endosperm protein extracted from expressing T_3 genotypes were subjected to densitometric analysis (not shown) that estimated a twelve-fold increase of the transgenic product compared to native LMW-GS with similar molecular weight. The higher amount of LMW-GS present in the transformed genotypes was confirmed by RP-HPLC separation of the polypeptides that remained insoluble after two extractions with 50% propanol (the polymeric or 'residue' protein fraction). Figure 3A shows the presence of a major peak (arrow) in the LMW-GS region from the transgenic seeds (dashed trace). SDS-PAGE (Figure 3B) confirms that this peak contains the transgenic LMW-GS. Comparison between the area corresponding to the transgenic LMW-GS and the area of a peak with similar elution time from non-transformed Bobwhite seeds (solid trace), showed that the transgenic LMW-GS is about 16-fold over-expressed in the residue fraction from the transgenic seeds. Because the residue is composed of the higher molecular weight glutenin polymers, it can be deduced that the transgenic polypeptide is included in this fraction. The ratio of the total residue protein in the transgenic *vs.* non-transformed seeds was calculated by dividing the total area under the dashed RP-HPLC trace in Figure 3 by the area under the solid trace. This ratio is 1.94. Thus, the over-expression of the transgenic LMW-GS results in a nearly two-fold increase of the amount of seed protein present in the polymeric fraction.

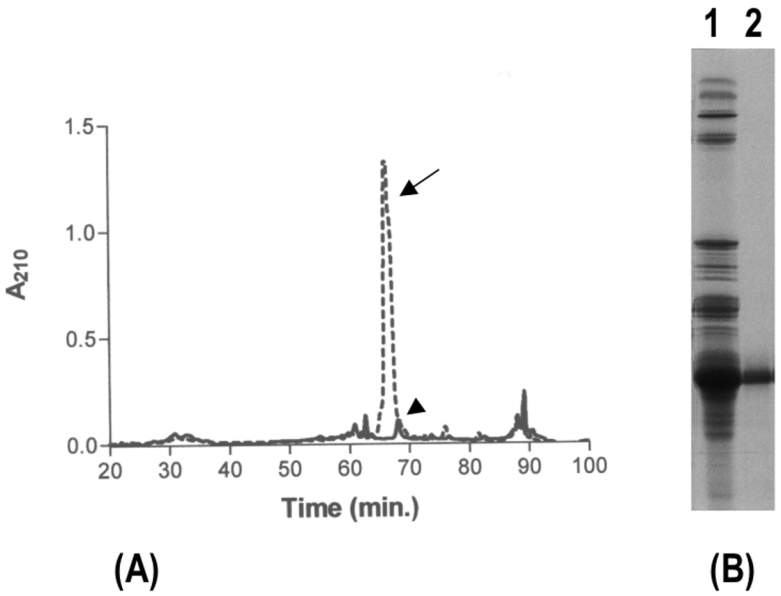


Figure 3. (A) Comparison between the RP-HPLC patterns of wild type Bobwhite (solid line) and the transformed genotype (dotted line). Arrow shows the peak corresponding to the transgene-encoded LMW-GS, as demonstrated by (B) SDS-PAGE analysis. Arrowhead indicates the LMW-GS peak of wild type Bobwhite used as a reference for over-expression quantification. (B) SDS-PAGE pattern of total seed protein from the transformed wheat cultivar (lane 1) and of the RP-HPLC fraction (indicated by the arrow in A) corresponding to the transgenic LMW-GS (lane 2).

Detection of the transgenic LMW-GS in the polymeric fraction

One and two-dimensional analyses of propanol soluble and insoluble fractions

These analyses were performed in order to evaluate the polymerization state of the transgenic LMW-GS. It is known that 50% propan-1-ol solubilizes gliadins and the oligomeric fraction of glutenins (Fu and Sapirstein 1996), whereas higher molecular weight glutenin polymers remain insoluble and are extractable only by using a reducing agent. Comparison of the SDS-PAGE patterns of soluble and insoluble fractions of seed proteins of the transgenic plants showed that the majority of the transgene-encoded LMW-GS is incorporated into the propanol-insoluble higher molecular weight glutenin polymers (Figure 4, lanes 5 and 6). A smaller amount is also evident in the soluble fraction after (Figure 4, lanes 3 and 4), but not before (lanes 1 and 2), reduction, indicating that the LMW-GS has been incorporated into oligomers. Indeed, such oligomers can be seen as bands in the soluble, unreduced, fraction from the transgenic seeds (dots, lanes 1 and 2) that are absent in the comparable fraction from the wild type Bobwhite seeds (Figure 4, BW, unreduced sample).

In order to detect possible disulfide linkages between the LMW-GS and other glutenins, two-dimensional electrophoresis (SDS-PAGE unreduced *vs*. SDS-PAGE reduced) was performed on the propanol-soluble fraction. For comparison, wild type Bobwhite was submitted to the same analysis. Figure 5 indicates the results of this comparison. HMW-GS are only present in trace amounts in the oligomeric fraction, as expected, since they are part of the highest molecular weight (insoluble) polymers (see also Figure 4, lanes 3-4). The transgenic LMW-GS is the predominant component in the oligomeric fraction (Fig 5A, arrow) and the lack of significant amounts of other glutenin subunit bands above or below the transgenic LMW-GS indicates that the latter is the predominant component of propanol-soluble oligomers.

Because polymeric proteins in the residue are too large to either be solubilized or to enter polyacrylamide gels (without reduction), they were first digested with the endoproteinase Lys-C, which internally cuts HMW-GS (Tao et al. 1992) and generates soluble polymers that are mainly composed of LMW-GS. Reduction of these polymers (Figure 6) shows that the transgenic LMW-GS is present in most of the polymers remaining after digestion (horizontal line at the

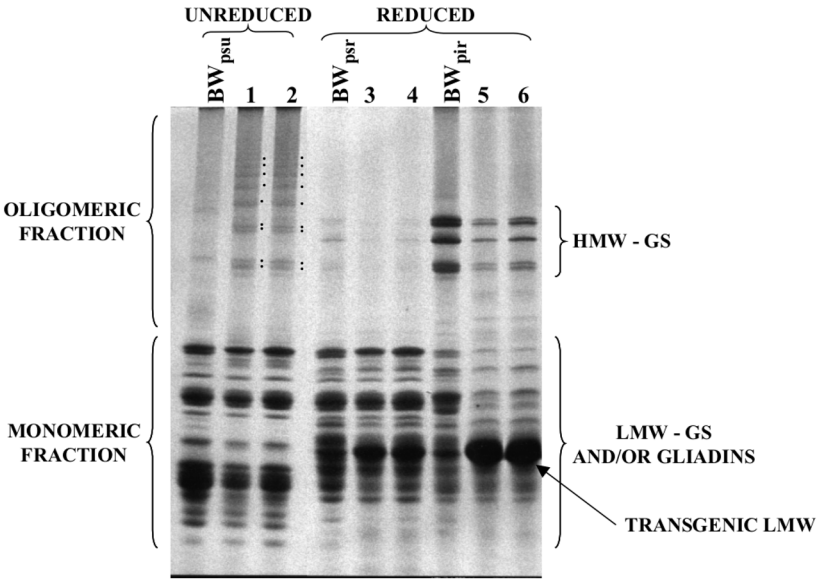


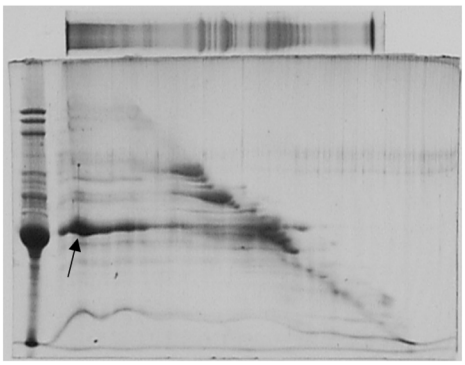
Figure 4. SDS-PAGE of seed proteins fractionated by propanol extraction. Unreduced propanol-soluble protein extracts from seeds of non-transformed Bobwhite (BW_{psu} = propanol-soluble unreduced) and transgenic samples (1, 2) are compared to their reduced counterparts (BW_{psr} = propanol-soluble reduced, 3 and 4, respectively), and to proteins from the reduced propanol-insoluble fraction (residue) of the same seeds: (BW_{pir} = propanol-insoluble reduced, 5 and 6, respectively). Bands corresponding in size to oligomers that were found in the transgenic samples, but not in the non-transformed control, are indicated with dots in lanes 1 and 2. Arrow designates the reduced transgenic polypeptide.

corresponding molecular weight present below the diagonal). Also, the nearly complete absence of endogenous LMW-GS indicates the predominance of the transgenic polypeptide in the larger LMW-GS polymers that remain after Lys-C digestion. However, the possibility of a low number of disulfide linkages between the transgenic and endogenous LMW-GS cannot be ruled out by this analysis.

Since analysis of Lys-C digestion products does not allow detection of possible linkages with HMW-GS, partial reduction was used as a second complementary method to solubilize the high molecular weight polymers in the residue fraction of the transgenic seed protein. Partial reduction releases the most accessible parts of the residue as lower molecular weight polymers (Werner et al. 1992; Lindsay and Skerritt 1998) that can be visualized by two-dimensional electrophoresis (unreduced followed by reduced SDS-PAGE). Four two-dimensional SDS-PAGE patterns corresponding to sequential extractions with four different reducing agent concentrations are shown in Figure 7. At lower DTT concentration (0.05 mM), the majority of the polymers released consist of large homopolymers of the transgenic LMW-GS, as indicated by the horizontal line of corresponding molecular weight on the left side of the gel. By increasing DTT concentration up to 0.5 mM, HMW-GS are visible on the same vertical lines as the transgenic LMW-GS on the very left side of the gel, showing the presence of very high molecular weight heteropolymers containing HMW-GS and/or the transgenic LMW-GS. At 1 mM DTT concentration, the amount of accessible monomeric transgenic LMW-GS increases, as shown by the presence of an intense spot along the diagonal, although there is still a predominance of polymers of different molecular weights (the horizontal line extends throughout the gel). HMW-GS are also present in trace amounts along the diagonal, but not visible below it, showing that at least the great majority of them is now present in the monomeric form. Finally, when the DTT concentration is 5 mM, the two-dimensional pattern resembles that of a fully reduced sample (usually performed at 60 mM DTT). Most of the spots are present along the diagonal, although a small number of lower molecular weight polymers containing the transgenic LMW-GS are still present.

SE-HPLC

To test whether over-production of the transgene-encoded LMW-GS changes the molecular size distribution of glutenin polymers, SE-HPLC was performed



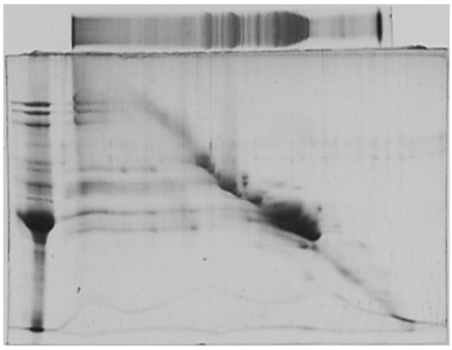


Figure 5. Two-dimensional SDS-PAGE (unreduced vs. reduced) analysis of the propanol-soluble fractions of (A) transgenic line and (B) wild type Bobwhite. For reference, a reduced sample of total seed proteins extracted from the transgenic genotype is shown on the left side of each gel, and the first dimension gels of unreduced propanol-soluble proteins are shown horizontally on the top of the second dimension gel. Arrow points to the monomeric form of the transgenic LMW-GS.

on both the soluble and insoluble (residue) fractions of expressor and non-expressor seeds from the same transgenic plant (Figure 8). The three replicates performed on each sample were perfectly overlapping. The chromatograms were divided into three areas, based on elution times: P_1, from 8 to 10 min; P_2, from 10 to 16 min; and P_3, from 16 to 21 min, roughly corresponding to glutenin polymers mainly containing HMW-GS and LMW-GS and more than 500,000 in molecular weight, smaller glutenin polymers or higher molecular weight monomers between 30,000 and 500,000 kDa, and oligomers or monomers smaller than 30,000 kDa, respectively. Areas under the trace for each division were measured and totalled and the ratios of those areas between expressors and

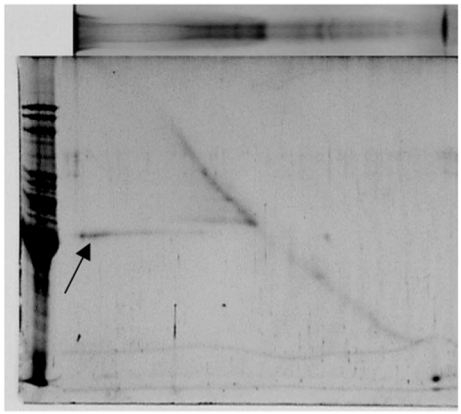


Figure 6. Two-dimensional SDS-PAGE (unreduced vs. reduced) analysis of the Lys-C digest of the residue proteins of a transgenic sample. The Lys-C digestion products are shown as a horizontal gel strip (first dimension) at the top of the second dimension gel. Arrow indicates the transgenic polypeptide. For reference, a reduced sample of the total seed proteins is shown on the left side of the gel.

non-expressors were calculated (Table 2). The total soluble fraction obtained from the expressor genotype (dashed line in Figure 8A) is 65% lower than that from the non-expressor. This decrease is mainly due to a reduction in proteins in the middle of the P2 fraction (Figure 8A, approximately 13 min). Conversely, the total area occupied by the insoluble fraction from the expressor genotype (Figure 8B) is 1.18 times larger than that of the non-expressor genotype, indicating that the expressors have a larger amount of glutenin polymers, and a lower content of smaller oligomers and gliadins, compared to the non-expressor. Polypeptides eluting in P_1 and P_2 are the most affected by the presence of the transgenic LMW-GS since their calculated ratios deviate the most from one. Because P_1 and P_2 are composed mainly of glutenin polymers, this confirms that the transgenic LMW-GS is part of the polymeric fraction.

SDS sedimentation test

The volume of sediment that falls at 1x*g* in 10 min after SDS solubilization of wheat flour is a rough indicator of the strength of doughs that can be made from that flour (Dick and Quick 1983). SDS sedimentation values found in expressor and non-expressor genotypes, along with their overall seed protein contents, are shown in Table 3. The transgenic genotype (expressor) shows a considerably lower SDS sedimentation value compared to the control, even though the former's seed protein content is

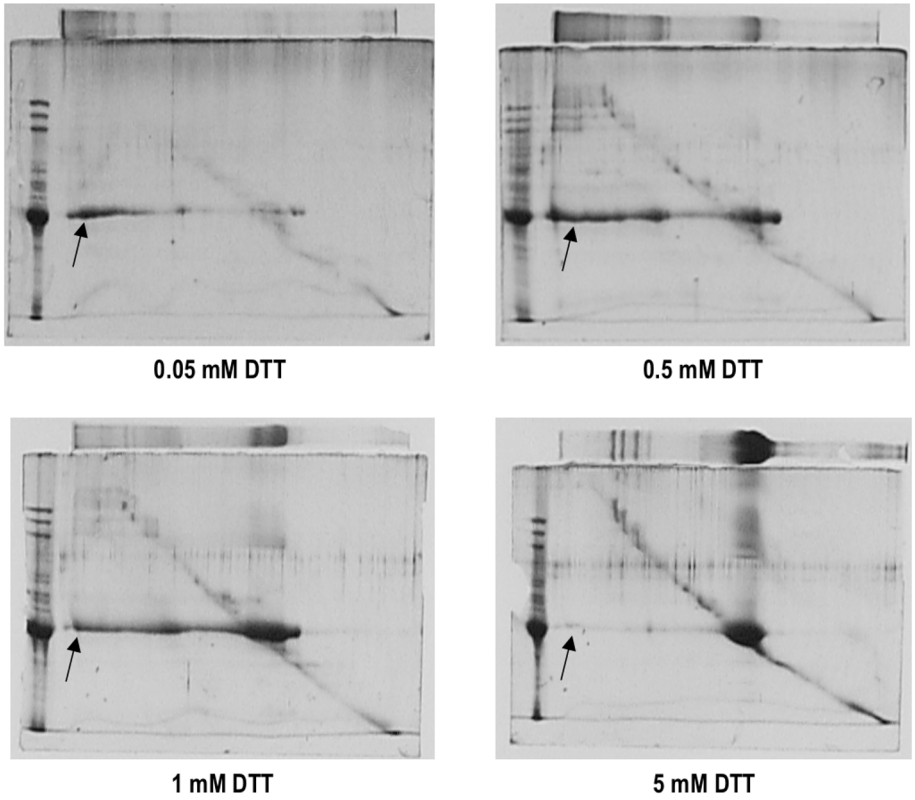


Figure 7. Two-dimensional SDS-PAGE (partially reduced followed by completely reduced) analysis of polymers released following sequential treatment of transgenic sample residues with differing DTT concentration (indicated below each gel). The partially reduced products are shown as a horizontal gel strip (first dimension) at the top of each second dimension gel. Arrows indicate the transgenic polypeptide. For reference, a reduced sample of total seed proteins extracted from the trangenic genotype is shown on the left side of each second dimension gel.

higher. Since increased protein content generally increases SDS sedimentation values, these results suggest that the quality of the protein in the LMW-GS transgene expressors, as measured in this test, has been changed.

Discussion

On the basis of the over-expression of the pLMWF23 transgene, we selected one transgenic wheat line for extensive characterization. The transgenic LMW-GS is estimated to be twelve or sixteen times over-expressed with respect to endogenous LMW-GS, depending on the method used (densitometric analysis of the SDS-PAGE patterns, or peak area in RP-HPLC traces) or on the fraction considered (total protein or glutenin fraction). This over-expression is likely due to insertion of multiple transgenes, as suggested by the high copy number of the fragment corresponding to the intact plasmid in Southern blot analysis.

The remaining ten BAR resistant lines were not further analyzed, although they might express the transgene at a low level. During the planning of our experiments, we anticipated the difficulties we might encounter in identifying transformants expressing the introduced LMW-GS transgene, because of the high number and sequence similarities of LMW-GS genes already present. Although a molecular tag could facilitate the identification of transgenic LMW-GS, we deliberatively avoided its use, both because we wanted to introduce a naturally-occurring LMW-GS, and because we decided to select only those transgenic plants with high expression levels of the transgenic LMW-GS. Our goal was to produce major changes in the glutenins/gliadins ratio to make it more likely that we could detect real effects of the

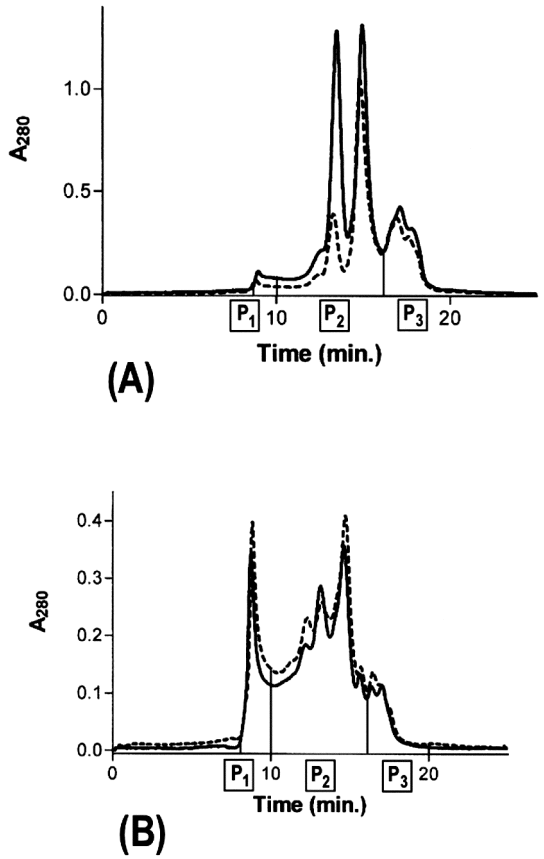


Figure 8. SE-HPLC patterns of the propanol-soluble (A) and insoluble (B) fractions of expressor (dashed lines) and non expressor (solid lines) genotypes. P_1, P_2, and P_3 define areas corresponding to different molecular weight ranges (see text).

Table 2. Ratios between the different SE-HPLC areas of expressor and non-expressor genotypes in the soluble and residue fractions

	Ratios between expressor and non-expressor: soluble proteins	Ratios between expressor and non-expressor: residue proteins
P_1	0.53	1.19
P_2	0.59	1.21
P_3	0.86	1.05
Total	0.65	1.18

LMW-GS and exclude the possible influences of the tag itself on gluten properties.

We observed a lower than expected number of expressor progeny in the T_2, T_3 and T_4 generations (Table 1). These observations could be indicative of transgene silencing or co-suppression in a subpopulation of the seeds, or of instability of the chromosome(s) carrying the transgenes. Gene silencing has been reported when multiple copies of introduced transgenes are integrated into the plant genome; transgene-mediated co-suppression can occur when the introduced genes are homologous to endogenous genes (Flavell 1994). To distinguish these possibilities would require assaying the progeny DNA for the presence of the LMW-GS transgene; such experiments would be difficult because of the presence of closely related endogenous LMW-GS genes. In some T_4 progeny, a high level of transgene over-expression was associated with lower levels of other storage proteins, including high molecular weight glutenin subunits or gliadins (data not shown). Since transgene mediated co-suppression only affects closely related genes, i.e., LMW-GS, this decrease may be the result of the diversion of the seed protein nitrogen reserves into making huge amounts of the transgenic LMW-GS at the expense of all the other seed proteins. Further investigations could identify the cause of such observations, but, for the purpose of the present study, we were interested mainly in determining the influence of the over-expression of the transgenic protein on glutenin polymer formation and, consequently, on gluten visco-elastic properties. Therefore, in the present work, we used only those individuals that over-expressed the transgenic LMW-GS, but did not show any detectable change in expression of the other storage proteins, as assessed by SDS-PAGE comparison to wild type Bobwhite seed proteins.

The analyses performed showed that the transgenic LMW-GS was incorporated into glutenin oligomers and polymers. In particular, two-dimensional analysis indicated that the transgenic polypeptide is the main component of oligomers that are propanol-soluble and of polymers that remain after Lys-C digestion. The absence of detectable endogenous LMW-GS in the gels might be due to their relative scarcity compared to the transgenic LMW-GS. It is likely that overloading the gels would reveal the presence of the endogenous LMW-GS.

Confirmation of the polymeric nature of the transgenic LMW-GS comes from the observation that it is present in both the most and least reduction-accessible regions of the polymer. Moreover, SE-HPLC peaks P_1 and P_2, which mainly contain glutenin polymers, are the most affected by its presence. According to SE chromatographic patterns, increasing the LMW-GS content in the transgenic genotypes is associated with a decrease in gliadin synthesis. This effect might indicate either differen-

Table 3. SDS sedimentation test values (Replicas I, II, III and average +/- standard deviation in mm) and seed protein content (%) in expressor and non-expressor genotypes

	Replica I	Replica II	Replica III	Average	Protein content
Expressor	55	55	55	55 ± 0	17.3%
Non-expressor	75	75	73	74.3 ± 1.15	15.7%

tial regulation of the levels of different proteins or a limitation on cellular resources for protein synthesis. Seeds of the expressor genotypes also had a lower amount of oligomers, perhaps because the high quantity of transgenic LMW-GS, in addition to increasing polymer amount, also increases polymer size by promoting cross-linking. It was not possible, however, to directly detect this, since the SE chromatography approach does not allow calibration of polymer sizes that are beyond the resolution limit of the gel filtration columns (Wrigley 1996).

It is not possible with the analyses performed here to determine whether the polymers containing the LMW-GS are homopolymers or also contain linkages with other LMW-GS or HMW-GS. In the partial reduction experiments, polymers containing HMW-GS are less accessible to reduction, as already reported by Lindsay and Skerritt (1998). Possible explanations for this are the higher number of glutenin subunits linked together, or their linking through a higher number of disulfide bonds (consequently they need a higher DTT concentration), or that they are found in the innermost parts of the glutenin polymer structure. The LMW-GS is found in some of these same fractions, indicating that it is either linked to HMW-GS or can form homopolymers of similar sizes or reduction-accessibility. On the basis of the great abundance of the transgenic LMW-GS, this latter possibility would involve a mechanism of segregation of the transgenic LMW-GS inside the cell from the endogenous glutenin subunits. In fact, if all glutenin subunits, included the transgenic one, are synthesized, processed and transported within the cell by the same mechanisms, it seems more likely than not that they link to each other, including HMW-GS, just on the basis of probability. Because there are so many transgenic LMW-GS molecules, formation of homopolymers or polymers dominated by the transgenic moiety might just be a statistical outcome of higher probability.

Our experiments are the first report of transformation of bread wheat with a LMW-GS gene and allow us to test the effect of an increase in the amount of this protein class on gluten visco-elastic properties. Previous work has indicated that transformation of wheat with HMW-GS genes modifies dough properties, as measured by mixographic and rheological analyses and baking tests. Effects varied, depending on the type of subunit and the recipient genotype. Two types of HMW-GS have been used to transform both bread and durum wheat, namely 1A*x*1 (He et al. 1999; Popineau et al. 2001; Vasil et al. 2001) and 1D*x*5 (Popineau et al. 2001; He et al. 1999; Rooke et al. 1999). When the levels of expression of the transgenic 1A*x*1 or 1D*x*5 subunits were comparable to those of endogenous subunits, an increase in dough strength was observed. In all experiments, the presence of transgenic 1Dx5 resulted in either an over-strong dough (He et al. 1999; Rooke et al. 1999), or in an inability to form a cohesive dough (Popineau et al. 2001). The behaviour of doughs containing elevated levels of Dx5 could be due to the presence of the extra cysteine residue, compared to the other *x*-type subunits, that very likely increases cross-linking.

In comparison to HMW-GS, the smaller size of the repetitive domain and the different cysteine distribution in LMW-GS could be expected to contribute in a different manner to the glutenin architecture and, consequently, to gluten visco-elastic properties. Since the pLMWF23A-encoded LMW-GS is predicted to be a chain extender, because of the presence of two cysteines that are available for forming intermolecular disulfide bonds, its cross-linking performance might be similar to that of HMW-GS 1A*x* (He et al. 1999; Popineau et al. 2001; Vasil et al. 2001). At the same time, glutenin polymers containing the transgenic LMW-GS could be smaller than those formed by HMW-GS, thereby allowing a finer tuning of glutenin polymer size. However, the lower sedimentation volumes of the transgenic flour measured by the SDS sedimentation test (indicative of lower gluten strength), show that over-expression of the transgenic LMW-GS, even when found in a higher protein seed, negatively affects gluten visco-elastic properties. These data can have at least two possible interpretations. They could suggest that the existing correlations between the size and/or amount of glutenin

polymers and quality parameters are not valid under all conditions. Alternatively, the low SDS sedimentation value might be due to the presence of homopolymers of the transgenic LMW-GS, whose size, although beyond the resolution limit of SE-HPLC, is not large enough to increase the ratio between the very large and the smaller polymers. If the former hypothesis is valid, then reducing the level of expression of the transgenic LMW-GS, either by decreasing the number of active genes (through segregation, if the multiple insertions are not located at closed linked loci) or by finding transformants with lower expression of the transgene, might result in improved strength. However, our results demonstrate that gluten polymer composition can be altered by overexpression of a LMW-GS and that such changes affect wheat end-use properties.

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